

1 **Impaired blood neutrophil function in the frequent exacerbator of chronic**
2 **obstructive pulmonary disease: a proof of concept study**

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14 **Abbreviations:**

15 α 1-PI, alpha 1- proteinase inhibitor; CL, chemiluminescence; eCO, exhaled carbon
16 monoxide; fMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balanced salt
17 solution; NADPH, nicotinamide adenine dinucleotide phosphate; NE, neutrophil
18 elastase; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species;
19 RLU, relative light units; TLR, Toll-like receptor.

Abstract

Purpose The underlying biological mechanisms of the frequent exacerbator phenotype of COPD remain unclear. We compared systemic neutrophil function in COPD patients with or without frequent exacerbations.

Methods Whole blood from COPD frequent exacerbators (defined as ≥ 2 moderate-severe exacerbations in the previous 2 years), and non-exacerbators (no exacerbations in the preceding 2 years) was assayed for neutrophil function. Neutrophil function in healthy ex-smoking volunteers was also measured as a control (reference) group.

Results A total of 52 subjects were included in this study; 26 frequent exacerbators, 18 non-exacerbators and 8 healthy controls. COPD frequent exacerbators had blunted blood neutrophil fMLP-stimulated oxidative burst compared to both non-exacerbators ($p < 0.01$) and healthy controls ($p < 0.001$). There were no differences between COPD frequent exacerbators and non-exacerbators in blood neutrophil PMA-stimulated oxidative burst but both COPD groups had reduced responses compared to healthy controls ($p < 0.001$). Bacterial-stimulated neutrophil degranulation was greater in frequent exacerbators than non-exacerbators ($p < 0.05$).
Conclusion This study is the first to report aberrant receptor-mediated blood neutrophil function in the frequent exacerbator of COPD.

Keywords: chronic obstructive pulmonary disease; elastase; granulocyte; polymorphonuclear leukocytes; reactive oxygen species.

41 **Introduction**

42 Chronic Obstructive Pulmonary Disease (COPD) is characterized by a progressive
43 decline in lung function and associated with chronic aberrant inflammatory responses
44 of the lung and airways to noxious stimuli [1]. Globally, COPD is now the third leading
45 cause of mortality [2], and the second leading cause of disability-adjusted life-years
46 lost [3]. Acute exacerbations (defined as sustained worsening of symptoms beyond
47 the normal day to day variation that may result in change of medical treatment and/or
48 hospitalisation) represent one of the primary manifestations of COPD and account for
49 50% to 75% of the costs associated with disease [4]. More frequent exacerbations
50 increase the risk of hospitalization, contribute to increased mortality risk during
51 hospitalisation and are associated with faster decline in lung function and worsening
52 health related quality of life [1,5,6].

53 The Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints
54 (ECLIPSE) study proposed that frequent exacerbators are a distinct phenotype in the
55 moderate-severe stages of the disease that is relatively stable over time [7,8]. More
56 specific phenotyping of COPD appears an increasingly important step towards
57 improving clinical management [8]. Despite clearer recognition of the frequent
58 exacerbator phenotype, the underlying biology of the susceptibility to exacerbations
59 remain unclear [9]. In order to better understand the pathogenesis of exacerbations,
60 comparisons of patients with none versus frequent exacerbations are required [10].
61 As a result of the identification of large alterations in immune related gene expression
62 within blood of frequent exacerbators [11], researching the pathophysiology of COPD
63 exacerbations by focusing on changes that occur in the systemic immune

64 compartment rather than a specific pulmonary immune defect *per se* could be fruitful.

65 Neutrophils, particularly neutrophil-derived proteinases, have been implicated in lung
66 destruction and remodelling and hence pathogenesis of COPD [12]. Neutrophil count
67 and neutrophilic inflammatory mediators are higher within the airways, even in the
68 stable (non-exacerbated) state in COPD [13]. Blood neutrophils also show an altered
69 pattern of activity in the stable state in COPD, with impaired chemotaxis and reduced
70 intracellular reactive oxygen species (ROS) production [14-16]. Evidence of further
71 systemic immune dysregulation occurs during exacerbations with circulating
72 neutrophils displaying up-regulation of inflammation related genes, enhanced
73 expression of cell adhesion molecules and elevated production of elastase and ROS
74 [17-20]. Despite an abundance of data supporting the hypothesis that neutrophils are
75 key effector cells in the development and progression of COPD [13], we could find no
76 studies assessing blood neutrophil function of COPD patients according to
77 exacerbation history.

78 To better establish the biological underpinning of the frequent exacerbator and
79 ultimately direct development of novel therapies for this high-risk group of patients,
80 we need an improved understanding of the changes in systemic immune function
81 associated with this phenotype. With this in mind, this proof of concept study was
82 designed to characterise blood neutrophil function of COPD patients in a stable state
83 with or without a history of frequent exacerbations. The primary aim of this study was
84 to test the null hypothesis that there is no difference in *in vitro* blood neutrophil
85 function between COPD frequent exacerbators, COPD non-exacerbators and healthy
86 controls.

87 **Methods**

88 **Study design and participants**

89 Following loco-regional ethics approval, 44 patients with COPD, defined as over 40
90 years, at least 10 pack year smoking history and post-bronchodilator (100 mcg of
91 inhaled salbutamol) FEV1<80% predicted with FEV1/FVC ratio<0.70 [1], were
92 prospectively recruited from outpatient clinics of a UK district hospital.

93 Current symptoms (cough, sputum), exacerbation history, co-morbidities, prescribed
94 medications and smoking history were collected at interview. Participants underwent
95 clinical examination and spirometry (Vitalograph Alpha[®], Vitalograph Ltd., UK).
96 Frequent exacerbators were patients who had 2 or more exacerbations requiring oral
97 corticosteroids and/or antibiotics during the last 2 years, and/or attendance to
98 hospital [1]. Patients were deemed non-exacerbators if they had not attended hospital
99 nor required systemic treatments for their COPD during the previous 2 years. Self-
100 reports were confirmed through hospital records and GP prescriptions. Eight healthy,
101 ex-smokers who had no symptoms of lung disease and had normal spirometry were
102 recruited as a control (reference) group. Informed consent was obtained from all
103 individual participants included in the study.

104 We excluded current smokers defined as anyone reporting smoking a cigarette within
105 6 months or having an exhaled carbon monoxide (eCO) >10 parts per million
106 (MicroCO, CareFusion Ltd.UK) on the day of testing. We also excluded anyone with
107 known structural lung disease (asthma, bronchiectasis, pulmonary fibrosis); cancer
108 (other than non-melanotic skin cancer); severe renal failure (calculated eGFR less

than 60 ml/min) or, liver failure; immunodeficiency or autoimmune conditions; anyone prescribed long-term antibiotics (including azithromycin), aminophylline, maintenance oral steroids, or other immunosuppressive medications. We included patients prescribed inhaled corticosteroids (ICS), anticholinergics and long- and short-acting beta agonists. All patients were prescribed optimal medication for their COPD according to current guidelines [1] and all were deemed clinically stable with none reporting a worsening of symptoms (no exacerbation) in the previous 3 months.

Sample collection and haematological analysis

Participants provided 10 ml of blood (K_3 EDTA and Lithium-Heparin) from the antecubital vein. Total and differential leukocyte counts and platelets were recorded on K_3 EDTA anticoagulated whole blood using an automated hematology analyser (ADVIA 2120, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Neutrophil assays

As described previously [21], neutrophil phorbol-12-myristate-13-acetate(PMA)- and formyl-methionyl-leucyl-phenylalanine(fMLP)-stimulated oxidative burst were assessed by a chemiluminescence (CL) kit (ABEL®04M, Knight Scientific Ltd, Plymouth, UK) incorporating the light-emitting protein Pholasin®. The CL per well was measured by a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Each well contained: 10 μ L of diluted whole (K_3 EDTA) blood (ratio of 1:100 with Hank's balanced salt solution; HBSS, without calcium and magnesium), 90 μ L assay buffer (HBSS with calcium and magnesium), 50 μ L Pholasin and 20 μ L adjuvant K. These mixtures were gently shaken and incubated at 37 °C for 30 s in

the luminometer, prior to the addition of 20 μ L of PMA (5 μ g/mL), 20 μ L fMLP (10 μ M) (or additional 20 μ L of HBSS for unstimulated wells) to provide an end total volume of 200 μ L per well, a 1:10¹⁰ final blood dilution and stimulated wells containing a PMA or fMLP concentration of 0.5 μ g/mL or 1 μ M. These concentrations have been standardised to be not rate limiting, even in the presence of abnormally high numbers of leukocytes, and thus provide responses that are reproducible. For PMA, CL of stimulated and unstimulated replicates of the same samples were recorded as relative light units (RLU) at 20 s intervals for 30 min. For fMLP, CL was recorded every second for 300 s. The area under the unstimulated CL curves was subtracted from stimulated curves of the same sample to determine PMA- or fMLP-stimulated oxidative burst. To calculate responses on a per cell basis in whole blood, area under the CL curve was expressed to number of neutrophils (in well) only as the contributions of monocytes, eosinophils and basophils in our 1:10¹⁰ final dilution of blood are considered to be insignificant [21,22].

Neutrophil stimulated degranulation was determined as previously described [23]. Heparinised blood (1 mL) was added to a microcentrifuge tube with 50 μ L bacterial stimulant containing *Staphococcus aureus*, *Psuedomonas fluorescens* and *Aerobacter aerogenes* (840-15, Sigma, Poole, UK). The microcentrifuge tubes were initially mixed by gentle inversion before incubation at 37 °C for 1 h (all tubes also mixed halfway through). Following incubation, the tubes were centrifuged for 2 min at 16 000 *g*, with the supernatant being immediately removed and stored at –80 °C until further analysis. Following thawing at room temperature, α 1-proteinase inhibitor(α 1-PI)/neutrophil elastase(NE) complex was measured in all samples using an ELISA kit (Calbiochem®, Merck, Darmstadt, Germany). Bacterial-stimulated degranulation was

based on subtracting α 1-PI/NE of unstimulated samples (heparinized plasma at same time point) away from stimulated samples and expression per neutrophil.

Statistical analysis

Statistical analysis was performed using SPSS (v21.00; SPSS Inc., Chicago, IL, USA). Normality was tested using the Shapiro-Wilk test and statistical significance was taken as $p < 0.05$. Primary outcome measures were stimulated neutrophil function. Data were analyzed between groups using one-way analysis of variance and independent t-tests or Kruskal-Wallis and Mann Whitney U tests. Relationship between baseline neutrophil count and daily beclamethasone dose with neutrophil function were assessed using Pearson correlation. For categorical data (gender) Fisher's exact test was applied.

Results

Clinical characteristics and blood leukocytes

Clinical details and total and differential leukocyte counts are summarised in Table 1. As expected FEV1 was significantly greater in healthy controls compared to both COPD groups ($p < 0.001$). No significant differences were found between COPD frequent exacerbators and non-exacerbators in FEV1, prescribed daily beclamethasone equivalent dose and leukocyte counts ($p > 0.05$).

Neutrophil oxidative burst

Both COPD frequent exacerbators and non-exacerbators had lower fMLP-stimulated oxidative neutrophil burst compared to controls, with frequent exacerbators also showing significantly lower function compared to non-exacerbators (Fig. 1).

Neutrophil PMA-stimulated oxidative burst was significantly lower in both COPD groups compared to controls (Fig. 2). However, there was no significant difference between frequent exacerbators and non-exacerbators COPD for responses to PMA ($p = 0.45$). To help determine the effect of baseline inflammatory status on subsequent neutrophil responsiveness, we investigated whether ICS exposure or baseline blood neutrophil count correlated with measures of neutrophil function. There were no correlations between fMLP-stimulated response and baseline neutrophil count ($p = 0.42$), or between fMLP-stimulated response and daily beclamethasone equivalent dose ($p = 0.17$). There were no correlations between PMA-stimulated oxidative burst and baseline neutrophil count ($p = 0.791$) or between PMA-stimulated responses and daily beclamethasone equivalent dose ($p = 0.30$).

Neutrophil degranulation

Blood neutrophils of COPD frequent exacerbators showed heightened bacterial-stimulated degranulation (α_1 -PI/NE complex) compared to non-exacerbator COPD (Fig. 3). Although there was no correlation between baseline neutrophil count and stimulated concentrations of α_1 -PI/NE complex ($p = 0.880$), there was a significant positive correlation between daily beclamethasone equivalent dose and bacterial-stimulated degranulation ($p = 0.04$, $r = 0.298$).

195 **Discussion**

196 In the present study, we showed that blood neutrophil oxidative burst is blunted in
197 COPD with receptor-dependent ROS production showing greater impairment in the
198 frequent exacerbator phenotype. Assessment of total degranulation responses of
199 blood neutrophils showed further dysregulation associated with the exacerbator
200 phenotype.

201 To the best of our knowledge, this is the first report of distinct patterns of neutrophil
202 function that relate to COPD exacerbation phenotype whilst in a stable state. We do
203 not prove mechanistically that changes in neutrophil effector functions are the causes
204 of or as a result of frequent exacerbations but provide an important starting point for
205 future investigations.

206 This study supports previously reported evidence of reduced intracellular oxidative
207 burst to fMLP in COPD populations compared to healthy counterparts [15, 24].
208 Impaired chemotactic responses to fMLP have also been observed in moderate-
209 severe COPD, compared to healthy smokers and non-smokers as well as COPD
210 patients with milder airflow obstruction [16]. These impaired functional responses to
211 stimulants are consistent with poor resistance to infection in COPD. Like us, these
212 other studies suggest greater severity of disease is not related with augmented
213 activity of inflammatory cells but a down-regulation. Our study, however, suggests
214 there are certain clinical phenotypes, which show further changes in neutrophil
215 responses to inflammatory stimuli (bacterial peptides) that may partly explain their
216 intrinsic susceptibility to recurrent infectious episodes. Blood neutrophils in COPD
217 demonstrate reduced migratory accuracy towards fMLP and decreased structural

changes and sensitivity to such chemotactic factors under receptor occupancy [14]. Aberrant blood neutrophil responses in COPD appear to be due to intrinsic cell defect (e.g. intracellular enzymatic reactions and kinases) rather than cell surface expression of chemoattractant receptors [14]. Further investigation (utilising whole blood flow cytometry) of immune regulation events upstream (e.g. at the level of FPR1 receptor) and downstream of neutrophil activation would help understand the interpretation and significance of impaired fMLP-stimulated oxidative burst in the frequent exacerbator.

Our findings of reduced blood neutrophil PMA-oxidative burst in COPD contrast with earlier reports of greater ROS production compared to controls [26, 27]. Differences in results could be explained by study participants (including the differences in treatment (e.g. non-ICS users in [26, 27]) or characteristics of the sampling and assays. For example, Renkema et al. [26] used heparinised samples (as opposed to EDTA), which have been demonstrated to interfere with neutrophils prior to subsequent activation of oxidative burst [28, 29]. Previous studies (26, 27) had also used isolation procedures that are known to influence neutrophil activation (including density gradient centrifugation, fluctuations in temperature) prior to any staining and *in vitro* stimulation. Our approach, using whole blood, provides minimal manipulation of cells and provides a more accurate representation of neutrophil behaviour *in vivo* (i.e. better maintenance of the extracellular milieu) [30]. In contrast to fMLP, we did not observe differences between exacerbation phenotypes in PMA-stimulated oxidative burst. Unlike the G-coupled receptor-dependent responses to fMLP, PMA penetrates the cell (independent of a receptor), triggering a long lasting, strong stimulation via protein kinase C and activation of NADPH oxidase throughout the cell.

PMA is considered an artificial stimulus (not encountered *in vivo*), differing substantially to physiological agonists (e.g. fMLP) [31]. Although PMA-stimulated responses provides further evidence of the aberrant intracellular signalling in COPD, such cell activation lacked biological sensitivity to characterise COPD phenotypes that may differ in their ability to recognise microbial moieties (e.g. formylated peptides) and mount responses toward infectious/inflammatory challenge [32].

COPD blood neutrophils possess exaggerated innate immune responses to Toll-like receptor (TLR) agonists (e.g. lipopolysaccharide) [33]. Upon triggering neutrophil degranulation, the concentration of free NE increases for only a brief period of time as its major inhibitors (e.g. α 1-PI) rapidly reach reaching equimolar concentrations [34]. Measurement of α 1-PI/NE complexes is considered to be marker of total NE release during neutrophil degranulation [35]. Here, we suggest that such heightened responses to TLR agonists (in our case both gram-positive and gram-negative bacteria) are more reflective of a COPD frequent exacerbator. These findings appear contradictory to the data on fMLP, whereby both fMLP-stimulated oxidative burst (e.g. FRP1) and bacterial-stimulated degranulation (e.g. TLR2, TLR4) represent receptor-mediated events. Similar differences between circulating phagocyte responses to chemoattractants and pro-inflammatory mediators have been observed in COPD [36]. Although distinct and complex intracellular transduction pathways are involved, one plausible mechanism for the observed variability between neutrophil effector functions in our study is the selective effect of ICS on one of the neutrophil function pathways (α 1-PI/NE). In COPD patients stratified according to GOLD severity, increasing ICS dosage was associated with enhanced stimulated neutrophil degranulation [37]. Budesonide and fluticasone propionate prolong human neutrophil

266 survival by inhibiting apoptosis at clinically relevant drug concentrations [38]. We
267 speculate that the greater (albeit non-significant) mean beclamethasone exposure of
268 the frequent exacerbators may partly explain the heightened NE release of
269 stimulated blood neutrophils in the frequent exacerbator.

270 Strengths of our study include its real-life setting using recognised clinical
271 phenotypes of COPD. Our findings are generalizable with patient demographics
272 reflecting patients with moderate-to-severe disease that attend a standard secondary
273 care service. The greater proportion of patients prescribed ICS in frequent
274 exacerbators corroborates previous findings [11] and reflects current treatment
275 guidelines [1]. Our number of participants is comparable to most other biological
276 studies comparing neutrophil responses in COPD. We also validated and controlled
277 for smoking status and used a healthy control group as an additional reference point.

278 The cross-sectional methodology does not allow us to identify whether responses of
279 the frequent exacerbator represent an intrinsic or acquired defect of neutrophil
280 function. The primary aim of this study was to characterise blood neutrophil function
281 (in the stable state) in COPD exacerbation phenotypes and not the temporal nature
282 of the relationship between inflammatory mediators and onset of COPD
283 exacerbations investigated previously [39]. We did not sample airway neutrophils to
284 compare against blood, however, others propose the dysregulation of immune
285 function in COPD frequent exacerbators may be systemic rather than a specific
286 abnormality limited to the lungs [12].

287 **Conclusions**

288 In conclusion, we have demonstrated aberrant blood neutrophil functions in COPD,
289 highlighting alterations in receptor-dependent responses that relate to the frequent
290 exacerbator phenotype. Frequent exacerbators have impaired oxidative responses to
291 chemotactic factors and augmented degranulation responses to bacterial triggers in
292 the circulation. Importantly, this study provides further support to a biological
293 underpinning of the frequent exacerbator phenotype and provides insights into
294 immune cell defects that can act as the basis for future investigations.

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297 their haematology analyser.

298 **Compliance with ethical standards**

299 **Conflict of interest:** KEL reports: grants, personal fees, non-financial support,
300 payments for attending Advisory Boards, speaker fees and reimbursements for
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302 payments for attending Advisory Boards, speaker fees and reimbursements for
303 attending conferences from AstraZeneca; personal fees, payment for attending
304 Advisory Boards and speaker fees from Pfizer; payment for attending Advisory
305 Boards from Teva, payment for attending Advisory Boards from Boehringer
306 ingelheim; all outside the submitted work. Authors declare that they have no other
307 potential conflicts of interest.

308 **Ethical approval:** All procedures performed in studies involving human participants
309 were in accordance with the ethical standards of the institutional and/or national
310 research committee and with the 1964 Helsinki declaration and its later amendments
311 or comparable ethical standards.

312

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420 **Table 1** Clinical characteristics of study participants.

Parameter	Healthy	COPD	COPD	p-value
	control	non-exacerbator	frequent exacerbator	
	(n = 8)	(n = 18)	(n = 26)	
Age, yrs	63.3 ± 7.6	68.7 ± 7.7	65.0 ± 7.5	0.15
Males / females, n	6/2	11/7	17/9	0.80
FEV ₁ , L	2.7 ± 0.4	1.0 ± 0.3	1.0 ± 0.5	<0.01
FEV ₁ , % predicted	89.7 ± 8.5	38.5 ± 10.8	36.6 ± 13.4	<0.01
Daily beclamethasone equivalent, µg		800 (0, 2000)	1500 (950, 2000)	0.14
Total leukocytes, 10 ⁹ ·L ⁻¹	7.0 (6.3, 7.7)	9.1 (6.9, 10.7)	7.1 (6.2, 8.9)	0.13
Neutrophils, 10 ⁹ ·L ⁻¹	4.0 (3.7, 4.7)	6.0 (4.3, 7.4)	4.6 (4.0, 6.3)	0.05
Monocytes, 10 ⁹ ·L ⁻¹	0.5 (0.4, 0.7)	0.5 (0.4, 0.6)	0.5 (0.3, 0.8)	0.70
Total lymphocytes, 10 ⁹ ·L ⁻¹	1.7 (1.6, 2.1)	1.7 (1.2, 2.2)	1.6 (1.0, 2.1)	0.51
Neutrophil: lymphocyte ratio	2.3 (1.8, 2.9)	3.6 (2.6, 5.1)	3.2 (2.0, 4.8)	0.06
Platelets, 10 ⁹ ·L ⁻¹	236 (221, 333)	284 (246, 389)	289 (238, 380)	0.53

421 Data are presented as mean ± standard deviation or median (interquartile range).

422 FEV1: forced expiratory volume in 1 s.

423 **Fig. 1** Neutrophil fMLP-stimulated oxidative burst (chemiluminescence) responses.
424 Columns indicate mean values for each group. Error bars represent standard
425 deviation. Significant difference between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

426 **Fig. 2** Neutrophil PMA-stimulated oxidative burst (chemiluminescence) responses.
427 Columns indicate mean values for each group. Error bars represent standard
428 deviation. Significant difference between groups: ** $p < 0.01$, *** $p < 0.001$

429 **Fig. 3** Bacterial-stimulated neutrophil degranulation ($\alpha 1$ -proteinase inhibitor/
430 neutrophil elastase). Columns indicate mean values for each group. Error bars
431 represent standard deviation. Significant difference between groups: * $p < 0.05$